G.V. Didenko
O.O. Krutz
V.N. Bazas
V.F. Chekhun

R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine, Kyiv, Ukraine

Keywords: *cancer vaccine, cytotoxic activity, tumor growth inhibition, Lewis lung carcinoma, Ehrlich carcinoma.*

DOI: 10.32471/oncology.2663-7928.t-24-1-2022-g.10377

EFFICIENCY OF XENOGENEIC ANTITUMOR VACCINE *IN VIVO*

Means of immunotherapy including cancer vaccines are believed to improve the outcome of cancer patients' treatment. In the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology a new cancer vaccine based on extracts of embryonic xenogeneic proteins combined with microbially-derived adjuvants is under investigation. ***Aim:*** *to study the anticancer efficacy and some immunological effects of the newly constructed xenogeneic cancer vaccine (XAV) in mice bearing Lewis lung carcinoma or Ehrlich carcinoma (EC).* ***Materials and methods:*** *the study was carried out on Balb/c and C57Bl mice transplanted ether with EC or Lewis lung carcinoma (LLC) respectively. Immunization with the XAV started shortly after the tumor cells transplantation (EC and LLC models) or after the primary tumor was surgically removed (LLC model). Anticancer and antimetastatic effects of the XAV were studied. Cytotoxic activity of lymphocytes, macrophages and blood serum was determined in MTT-assay.* ***Results:*** *immunization with XAV inhibited the growth of primary tumor in both experimental models, reduced metastatization in mice bearing LLC and significantly prolonged survival time of mice bearing EC. At the terminal stages of tumor growth, cytotoxic activity of lymphocytes and macrophages in vaccinated animals was elevated comparing with unvaccinated animals independently of experimental tumor.* ***Conclusion:*** *the newly designed xenogeneic cancer vaccine demonstrated promising anticancer efficacy and deserve further investigation.*

The limited success of the existing methods of cancer treatment (especially at the late stages) requires for the search and development of new more effective means of treatment. High hopes are put on biotherapy, which main objective is to develop methods for amplifying immune response of the body. To reach this goal, antitumor vaccines (AVs), co-stimulatory cytokines and molecules to enhance antitumor immune response are elaborating.

The autologous AVs which are constructed based on tumor materials of cancer patients are widely used in modern medicine. A common problem of such vaccines is that the tumor-associated antigens (TAAs) have low immunogenic activity [1]. The immunological potency of the vaccines can be enforced with the help of microbial-derived adjuvants. They can attract toll-like receptors on the surface of lymphocytes to enhance the immune response and thus they can stimulate the synthesis of cytokines.

Another approach to overcome immune tolerance and to induce immune response against endogenous proteins is the use of foreign counterparts of TAAs [2, 3]. As long as most TAAs are conserved, they bare high degree of homology between human proteins and their counterparts in different animal species [4]. Therefore, minor structural interspecies differences can be successfully used to induce an effective immune response to poorly immunogenic tumor antigens [5–7].

It is a well-known fact that cancer cells express oncofetal antigens. These include the proteins that are expressed at certain stages of embryonic development but are rarely detected on normal adult cells. The use of embryonic proteins as «universal» specific immunogens opens new possibilities for constructing AVs [8, 5].

In the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR) elaboration of a new approach to cancer vaccines development based on extracts of embryonic xenogeneic proteins combined with microbially-derived adjuvants is going on [9, 10]. The constructed xenogeneic AV (XAV) consists of chicken embryo proteins of high molecular weight and cytotoxic metabolite of *Bacillus subtilis* B-7025. The scope of the research was to study the anticancer efficacy and some immunological effects of the XAV in mice bearing Lewis lung carcinoma (LLC) or Ehrlich carcinoma (EC).

OBJECT AND METHODS

Experimental animals and tumor models. Research was carried out on C57Bl (10 animals per each group, males 2.5 months, 20–22 g, vivarium R.E. Kavetsky IEPOR National Academy of Science of Ukraine) and Balb/c mice (10 animals per each group, males, 2.5–3 months, 20–22 g, IEPOR vivarium). The use and care of experimental animals have been performed in accordance with the standard international rules on biologic ethics and was approved by Institutional Animal Care and Use Committee [11].

LLC and EC were used as experimental models.

Balb/c mice were injected with EC cells ($3 \cdot 10^5$ cells per animal) intramuscularly in the thigh hind limb.

C57Bl mice were transplanted with different doses of LLC depending on the further experiment they were planned to be used in.

In mice which were planned to be immunized after tumor challenge, LLC cells were injected intramuscularly in the thigh hind limb ($3 \cdot 10^5$ cells per animal).

In mice which were planned to be immunized after the surgical removal of the primary tumor, LLC cells were injected in the hind foot at a dose of $1 \cdot 10^5$ cells/mouse. On day 13 of tumor growth, when the primary tumor reached 0.6±0.2 cm in diameter, mice underwent surgical removal of the tumor. In brief, mice were injected with etaminal-sodium anesthesia (40 mg/kg of body weight), ligature above the site of operation was imposed and the tumor-bearing foot was removed. The wound was treated with iodine infusion.

Animals transplanted with LLC cells were divided on four groups:

1 — control group of tumor-bearing animals without surgery (control tumor-bearing);

2 — tumor-bearing animals treated with XAV (tumor + XAV);

3 — animals with removed tumor (RT);

4 — animals with RT treated with XAV (RT + XAV).

Experimental schedule. In LLC-bearing mice, vaccination started on the second day after tumor transplantation and continued on day 5; 12 and 19 of tumor growth (0.3 mg/ml in 0.3 ml per animal per injection).

In mice which underwent LLC-tumor resection, vaccination started on the second day after tumor removal (day 15 of the tumor growth) and continued on day 17; 24 and 31 after tumor transplantation.

On day 34 after tumor transplantation, all the animals were devitalized and subjected to immunological investigation.

In mice transplanted with EC-tumor, immunization schedule was the same as for the LLC-bearing mice: it started on the second day after tumor transplantation and continued on day 5; 12 and 19 of tumor growth. The XAV was injected in a dose of 0.3 mg/ml per animal per injection. On day 24 of tumor growth, all experimental mice were devitalized and material for immunological studies was taken.

XAV preparation. The XAV was constructed and studied at R.E. Kavetsky IEPOR, National Academy of Science of Ukraine. The XAV is composed of chicken embryonic proteins of high molecular weight extracted with EDTA (2% in Tris buffer, 1 h) [12], and cytotoxic metabolite of *B. subtilis* B-7025 [13]. The concentration of embryonic proteins and cytotoxic metabolite of *B. subtilis* B-7025 in the vaccine solution is 0.3 mg/ml for both components.

Methods of experimental oncology. In LLC-bearing mice, on day 34 of tumor growth the primary tumor was removed and weighted. The lungs of all LLC-transplanted mice were removed and checked for the metastases. The level of lung metastases was determined by counting the metastases number.

In EC-bearing mice the tumor diameter was measured by the calipers and the tumor volume was calculated by the formula: $V={}^1/_6 \cdot \pi d^3$, where d — the diameter of the tumor nodule.

The survival rate of animals was determined by the percentage of alive animals in the group to the total number of animals in the group at the beginning of the experiment.

The mean survival time of animals in each group was determined by the survival time of each mouse in a group.

Analysis of immune cells cytotoxic activity. The functional activity of immune cells was evaluated in MTT-assay [14, 15]. The target cells (either LLC or EC cells, in concentration of $3 \cdot 10^5$ cells in 100 µl of culture medium) and immune cells (either Lph or Mph in concentration of $1 \cdot 10^6$ in 100 µl of culture medium) were placed in 96-wells flat-bottomed plates and incubated for 18 h in 5% CO^2, 100% of humidity, 37 °C. MTT (Sigma, USA) in concentration of 5 mg/ml was added in quantity of 0.02 ml per well and incubation continued for 4 hours more under similar conditions, after that plates were washed twice with NaCl. To resolve formazan granules, 0.12 ml (2 mol/L) of KOH and 0.14 ml of 50% DMSO solution were added to the sediment. Optical density was measured at $\lambda = 545$ nm *vs* $\lambda = 630$ nm with a micro ELISA reader (StatFax-2100, USA). All samples were in triplicate. Cytotoxic Activity Index (%) was calculated by the formula:

$$(((A_i+A_t)-A_{i+t})/A_{i+t}) \cdot 100\%, \text{ where}$$

A_i — absorbance in the wells containing only immune cells (Lph or Mph);

A_t — absorbance in wells containing only target cells;

A_{i+t} — absorbance the in wells where immune cells and tumor target cells were added.

Analysis of blood serum cytotoxic activity. Cytotoxic activity of blood serum was studied in MTT-assay. To the preincubated target cells ($1 \cdot 10^6$ cells in 100 µl of culture medium) 10 ml of autologous blood serum was added. Plates were incubated for 18 h in 5% CO^2, 100% of humidity, 37 °C. All the next steps were the same as is described above.

Cooperative cytotoxic activity of lymphocytes and macrophages. The target cells (either LLC or EC cells, in concentration of $3 \cdot 10^5$ cells in 100 µl of culture medium) and both types of immune cells (Lph AND Mph in concentration of $1 \cdot 10^6$ in 100 µl of culture medium) were placed in 96-wells flat-bottomed plates and incubated for 18 h in 5% CO^2, 100% of humidity, 37 °C. All the next steps were the same as is described above.

Zymozan induced respiratory burst of Mph was studied in NBT-assay [16]. In brief, Mph ($1 \cdot 10^6$ cell/ml, 0.2 ml/well) were incubated with 0.02 ml/well of 0.2% NBT solution (Sigma, USA) and 0.02 ml/well of zymozan (100 µg/ml, Sigma, USA). After incubation (1 h, 5% CO_2, 37 °C), the plates were washed for two times with 0.9% NaCl solution. The 2 mole/liter KOH solution (0.06 ml/well) and 50% DMSO solution (0.07 ml/well) were used to dissolve diformazan granules. Optical density was measured at $\lambda =$ 630 nm with the use of micro ELISA reader (StatFax-2100, USA). Each sample was measured in triplicate. Index of potentiation (IP) was calculated as following:

$$IP = OU_{(induced\ test)} / OU_{(spontaneous\ test)}, \text{ where}$$

$OU_{(induced\ test)}$ and $OU_{(spontaneous\ test)}$ stand for optical units of wells with zymozan treated and untreated Mph respectively.

Statistical analysis. The statistical analysis was made using Student *t*-test. The difference was considered as significant when $p<0.05$. Calculations and graph plotting were performed with the OriginLab soft-ware.

RESULTS AND DISCUSSION

Study of the XAV efficiency in animals with LLC. Animals with LLC which were administrated XAV have smoler size of the primary tumor and significant decrease in metastases development. Data of the XAV anticancer efficiency in the groups are presented in Table 1.

Table 1

Antitumor efficiency of the XAV in animals with LLC on day 34 of tumor growth

Group	Mass of primary tumor, g	Rate of metastasis,%	Number of metastases, n
Control tumor-bearing	0.7 ± 0.1	100.0	34.1 ± 7.9
Tumor + XAV	0.1 ± 0.1*	30.0	1.2 ± 0.9*
RT	Primary tumor was removed	82.0	3.6 ± 0.8
RT + XAV	Primary tumor was removed	12.0	0.12 ± 0.1*

Note: *p <0.05 comparing with the corresponding control group.

Studying the immunological parameters, it was found that at terminal stages of tumor growth the level of Lph and Mph cytotoxic activity in vaccinated animals was elevated comparing with unvaccinated animals (Table 2).

Table 2

Cytotoxic activity of lymphocytes and macrophages in animals with LLC on day 34 of tumor growth

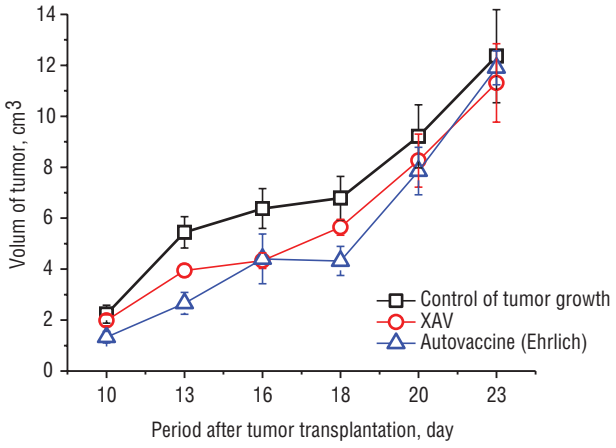
Group	Cytotoxic Activity Index,%	
	lymphocytes	macrophages
Control tumor-bearing	24.8 ± 0.6	25.7 ± 1.2
Tumor + XAV	36.0 ± 1.3*	35.8 ± 2.1*
RT	26.2 ± 4.5	29.7 ± 2.3
RT + XAV	29.3 ± 3.8	42.5 ±1.7*

Note: *p <0.05 comparing with the corresponding control group.

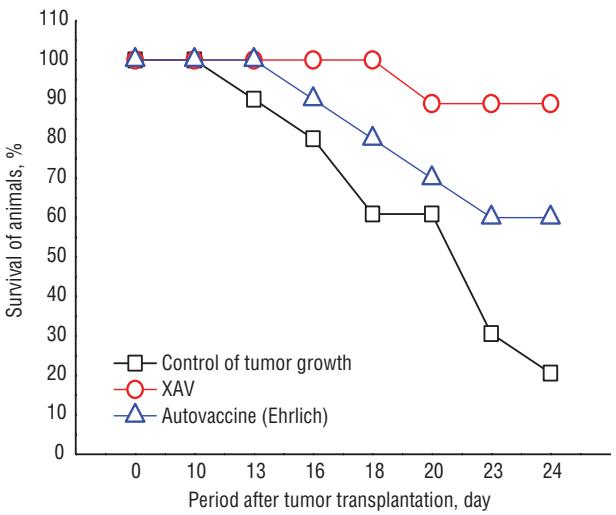
Thus, application of XAV in operated and non-operated animals with LLC significantly inhibited tumor growth and reduces the level of lung metastasis. Possibly, the XAV antitumor effect is ensured by the elevated functional activity of lymphocytes and macrophages which lasted till the late stages of tumor development.

Study of the XAV efficiency in animals with EC. The vaccination scheme was the same as in the application of XAV in animals with LLC: it started on the second day after the tumor transplantation and continued on day 5, 12 and 19 of tumor growth.

According to the results of the experiment, the XAV has antitumor effect comparable with the vaccine prepared from extracts of Ehrlich cancer cells: tumor volume in mice of both groups was significantly smaller as compared with unvaccinated tumor-bearing control till the day 20 of tumor growth (Fig. 1*a*). Although tumor volume of mice immunized ether with XAV or Ehrlich-cells-based vaccine were almost similar, the survival rate of former group of mice was significantly higher as compared to the latter one (Fig. 1*b*). For example, on day 24 of tumor growth 90% of XAV-vaccinated mace were still alive while in the other vaccinated group this index made only 60%. This may point to the better life quality of the XAV-vaccinated mice.



a



b

Fig. 1. The kinetics of tumor growth (*a*) and survival (*b*) of mice bearing EC who were administered the Erlich-cells-based vaccine or XAV

To address the mechanisms which may underlie XAV anticancer effect some immunological parameters of the XAV-vaccinated mice were analyzed. It was found that the antitumor efficiency of XAV may be related to activation of Lph and Mph cytotoxic activity. To illustrate, cooperative cytotoxic activity of Lph and Mph of immunized mice was approximately three times higher than in the group of control tumor-bearing mice (Fig. 2).

In XAV-vaccinated mice, blood serum cytotoxic activity was elevated too as compared to control tumor-bearing mice (Fig. 3).

Moreover, the respiratory burst of Mph in the vaccinated mice remained unaffected while in the group of control tumor-bearing mice it fallen almost 2 times beneath the level of the intact control mice (Fig. 4). This points to the Mph exhaustion or repolarization towards M2-like phenotype in the control tumor bearing mice.

So, the chicken embryo proteins-based vaccine administered together with *Bacillus subtilis B-7025* cytotoxic metabolite demonstrated its significant antitumor efficacy. Probably, this efficiency relies on macrophages and lymphocytes stimulation.

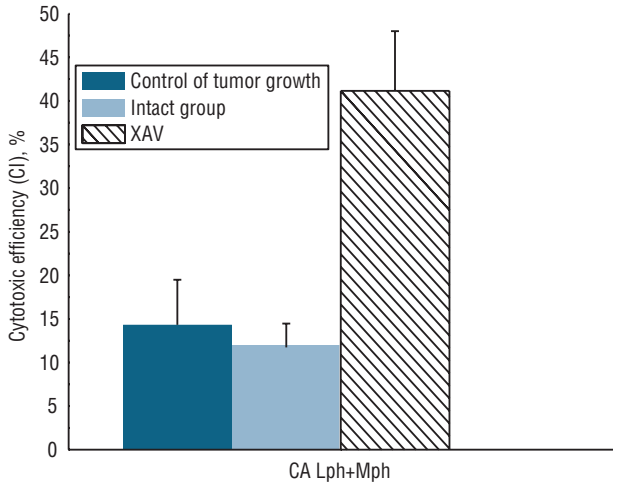


Fig. 2. Cooperative cytotoxic activity of lymphocytes and macrophages in control and XAV-vaccinated mice bearing EC on day 24 of tumor growth

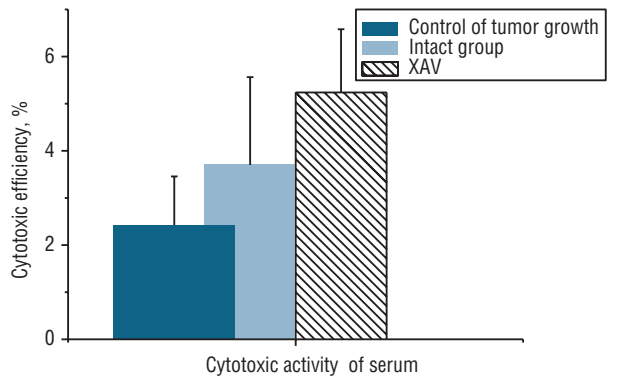


Fig. 3. The blood serum cytotoxic activity of control and XAV-vaccinated mice bearing EC on day 24 of tumor growth

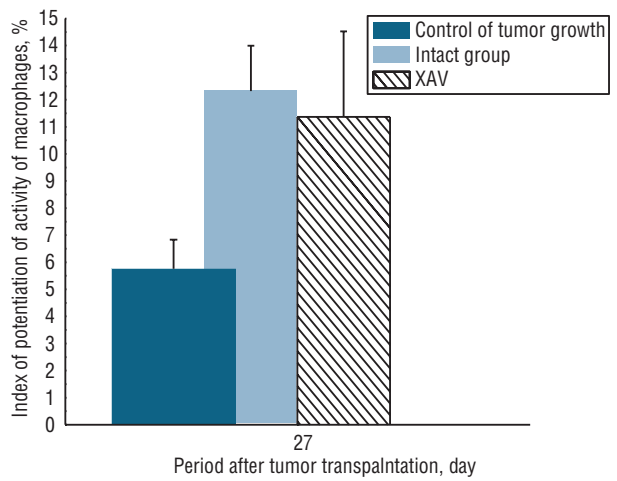


Fig. 4. Index of Mph respiratory burst potentiation after stimulation with zymosan in NBT-test

The utilization of embryonic tissue homogenates in antitumor therapy began in the 30s of the XX century. G. Ficher, *et al*. demonstrated antitumor efficacy of extracts derived from fetal liver and thymus [17]. Also, it was shown that the administration of embryonic tissue to rats 2–3 weeks before Jensen sarcoma transplantation leads to tumor rejection. Nowadays the scientific society continues working on the use of drugs based on embryonic tissue in antitumor therapy and have received quite encouraging results. Animal embryos can be used for manufacturing such vaccines [18]. Preliminary works in our Institute demonstrated that chicken embryo proteins possess remarkable anticancer and immunomodulating activities and can be used in cancer vaccines engineering [19]. One of the possible ways to improve vaccine efficacy is to combine it with immunological adjuvant.

The products of *Bacillus subtilis* are under the study of R.E. Kavetsky Institute since first half of the XX century [20]. For example, some proteins of *Bacillus subtilis B*-7025 were shown to possess cytotoxic and immunomodulating activities and therefore are feasible to be used in the construction of cancer vaccines. These cytotoxic proteins already have been used for construction of autologous vaccines [21, 22]. In this study, the cytotoxic protein of *B. subtilis B-7025* is used as the adjuvant in the AV based on xenogeneic embryo proteins.

Newly constructed embryonic vaccine was very effective in the treatment of animals with model tumors (LLC and EC), which was manifested in the delaying of primary tumor progression and in suppression of metastasis. Antitumor effect of the vaccine was shown to relay on activation and maintaining of immune cells (lymphocytes and macrophages) functional activity.

CONCLUSIONS

1. The AV based on the separate faction of embryo extract (proteins with molecular weight above 50 kD) and cytotoxic metabolite of *B. subtilis B-7025* have an inhibitory effect on the progression of primary tumor node and the development of metastases that was demonstrated on different models of tumor growth.

2. Antitumor effect of the XAV was shown to be based on the elevated activity of immune cells (lymphocytes and macrophages).

REFERENCES

1. **Moingeon P.** Cancer vaccines. Vaccine 2001; **19** (11–12): 1305–26. doi: 10.1016/s0264–410x(00)00372–8

2. **Naftzger C, Takechi Y, Kohda H**, ***et al.*** Immune response to a differentiation antigen induced by altered antigen: A study of tumor rejection and autoimmunity. Proc Natl Acad Sci USA 1996; **93** (25): 14809–14. doi: 10.1073/pnas.93.25.14809

3. **Wang M, Xie Z, Shi M, *et al.*** A new strategy to induce effective antitumor response in vitro and in vivo. Scand J Immunol 2008; **68** (3): 287–96. doi: 10.1111/j.1365–3083.2008.02140.x

4. **Erenprace YG.** Embryonic properties of tumor cells: facts and hypotheses. Exp Oncol 1982; **4** (6): 13–8.

5. **Sioud M, Sorensen D.** Generation of an effective anti-tumor immunity after immunization with xenogeneic antigens. Eur J Immunol 2003; **33** (1): 38–45. doi: 10.1002/immu.200390005

6. **Potebnya GP, Symchych TV, Lisovenko GS.** Xenogenic cancer vaccines. Exp Oncol 2010; **32** (2): 61–5.

7. **Su J-M, Wei Y-Q, Tian L, *et al.*** Active immunogene therapy of cancer with vaccine on the basis of chicken homologous matrix metalloproteinase-2. Cancer Res 2003; **63** (3): 600–7.

8. **Kochenderfer JN, Gress RE.** A comparison and critical analysis of preclinical anticancer vaccination strategies. Exp Biol Med 2007; **232** (9): 1130–41. doi: 10.3181/0702-MR-42

9. **Kuzmenko AP, Didenko GV, Shpak GE, Potebnya GP**. Comparative evaluation of the antitumor efficacy of allogeneic and xeno-

geneic vaccine modified by protein metabolites of *B. subtilis* B-7025. Clin Oncol 2014; **4** (16): 90–4. (in Ukrainian).

10. **Potebnya GP, Didenko GV, Kuzmenko OP, *et al.*** A method of producing antitumor vaccine (Pat №77647 UA). Publ 16.07.2012. Bul №4. (in Ukrainian).

11. **Kozhemyakin YuM, Kchromov OS, Filonenko MA, *et al.*** Guideline on management of laboratory animals and work with them. K: Avicenna, 2002; 156 p. (in Ukrainian).

12. **Ras M, Girbal-Neuhauser E, Paul E, Lefebvre D.** A high yield multi-method extraction protocol for protein quantification in activated sludge. Bioresour Technol 2008; **99** (16): 7464–71. doi: 10.1016/j.biortech.2008.02.025.

13. **Didenko GV, Sorokina LV, Shpak EuG, *et al.*** The application of fullerene C60 for the modification of an anticancer vaccine based on metabolism products of Bacillus subtilis 7025. J Biol Phys Chem 2011; **11**: 30–5.

14. **Ohno M, Abe T.** Rapid colorimetric assay for the quantification of leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). J Immunol Methods 1991; **145** (1–2): 199–203. doi: 10.1016/0022–1759(91)90327-c

15. **Kristensen E, Reimann R.** Direct cytotoxicity test applied to patients in stage I and II of malignant skin melanoma. Cancer Immunol Immunother 1977; **2**: 49–55.

16. **Goncharov AH, Frejdlin ES, Cmirnov VS, *et al.*** Basics of clinical immunology and methodological approach to immune status evaluation: manuals. Kaliningrad: Kaliningrad Univ, 1997: 51. (in Russian)

17. **Luo Y, Wen YJ, Ding ZhY, *et al.*** Immunotherapy of tumors with protein vaccine based on chicken homologous Tie-2. Clin Cancer Res 2006; **12** (6): 1813–9. doi: 10.1158/1078–0432.CCR-05–1990

18. **Biava PM, Fiorito A, Negro C, Mariani M.** Effect of treatment with embryonic and uterine tissue homogenates on Lewis lung carcinoma development. Cancer Lett 1988; **41** (3): 265–70. doi: 10.1016/0304–3835(88)90287-x

19. **Symchych TV, Fedosova NI, Karaman OM, *et al.*** Cancer xenogeneic vaccines based on chicken antigens. Why not? Oncology 2021; **23** (1–2): 11–7. doi: 10.32471/oncology.2663–7928.t-23–1-2021-g.9368

20. **Zatula DG.** Experimental and clinical results of application of antitumor vaccines obtained by means of bacterial metabolism. Neoplasma1984; **31** (1): 65–74.

21. **Didenko GV.** The designing of antitumor vaccines on the basis of protein-containing metabolites of *B. subtilis B-7025* and their effects on the separate reactions of anti-tumor immunity (experimental study). Kyiv, 2008. 19 p. (in Ukrainian).

22. **Potebnya G, Cheremshenko N, Lisovenko G, *et al.*** Antitumor efficacy of autovaccines prepared from chemoresistant tumor cells with the use of lectin of B. subtilis B-7025. Exp Oncol 2007; **4** (29): 277–80.

ЕФЕКТИВНІСТЬ КСЕНОГЕННОЇ ПРОТИПУХЛИННОЇ ВАКЦИНИ *IN VIVO*

Г.В. Діденко, О.О. Круць, В.М.Базась, В.Ф. Чехун

Інститут експериментальної патології, онкології і радіобіології ім. Р.Є. Кавецького НАН України, Київ, Україна

Резюме. *Засоби імунотерапії, включаючи протипухлинні вакцини, можуть підвищувати результати лікування хворих на рак. В інституті експериментальної патології, онкології і радіобіології ім. Р.Є. Кавецького НАН України проводяться дослідження ефективності нової протипухлинної вакцини на основі екстрактів ембріональних ксеногенних білків у поєднанні з ад'ювантами мікробного походження.* ***Мета:*** *вивчення протипухлинної ефективності та деяких імунологічних ефектів нової ксеногенної протипухлинної вакцини (КПВ) у мишей з карциномою легені Льюїс або карциномою Ерліха.* ***Об'єкт і методи****: дослідження проводили на мишах ліній Balb/c та C57Bl, яким відповідно були перещеплені клітини карциноми Ерліха (КЕ) або карциноми легені Льюїс (КЛЛ). Імунізацію за допомогою КПВ проводили безпосередньо після трансплантації пухлинних клітин (на моделі КЕ та КЛЛ) або після хірургічного видалення первинної пухлини (на моделі КЛЛ). Досліджено протипухлинний та антиметастатичний ефекти КПВ. Для визначення цитотоксичної активності лімфоцитів, макрофагів та сироватки крові використовували МТТ-тест.* ***Результати:*** *імунізація за допомогою КПВ призводила до уповільнення росту первинних пухлин незалежно від експериментальної моделі, пригнічення метастазування у мишей з КЛЛ та суттєвого подовження тривалості життя мишей з КЕ. На термінальних стадіях пухлинного росту цитотоксична активність лімфоцитів та макрофагів у вакцинованих тварин перевищувала таку у невакцинованих тварин незалежно від експериментальної пухлини.* ***Висновки:*** *розроблена ксеногенна протипухлинна вакцина продемонструвала протипухлинну і антиметастатичну активність та заслуговує на подальші дослідження.*

Ключові слова: протипухлинна вакцина, цитотоксична активність, гальмування росту пухлини, карцинома легені Льюїс, карцинома Ерліха.

Адреса для листування:
Діденко Г.В.
03022, Київ, вул. Васильківська, 45
Інститут експериментальної патології, онкології і радіобіології ім. Р.Є. Кавецького НАН України
E-mail: gennadij_d@ukr.net

Одержано: 24.11.2021